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Pathogen-induced ERF68 regulates hypersensitive cell death in tomato

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SUMMARY

Ethylene response factors (ERFs) are a large plant-specific transcription factor family and play diverse important roles in various plant functions. However, most tomato ERFs have not been characterized. In this study, we showed that the expression of an uncharacterized member of the tomato ERF-IX subgroup, *ERF68*, was significantly induced by treatments with different bacterial pathogens, ethylene (ET) and salicylic acid (SA), but only slightly induced by bacterial mutants defective in the type III secretion system (T3SS) or non-host pathogens. The ERF68-green fluorescent protein (ERF68-GFP) fusion protein was localized in the nucleus. Transactivation and electrophoretic mobility shift assays (EMSAs) further showed that ERF68 was a functional transcriptional activator and was bound to the GCC-box. Moreover, transient overexpression of *ERF68* led to spontaneous lesions in tomato and tobacco leaves and enhanced the expression of genes involved in ET, SA, jasmonic acid (JA) and hypersensitive response (HR) pathways, whereas silencing of *ERF68* increased tomato susceptibility to two incompatible *Xanthomonas* spp. These results reveal the involvement of ERF68 in the effector-triggered immunity (ETI) pathway. To identify ERF68 target genes, chromatin immunoprecipitation combined with high-throughput sequencing (ChIP-seq) was performed. Amongst the confirmed target genes, a few genes involved in cell death or disease defence were differentially regulated by ERF68. Our study demonstrates the function of ERF68 in the positive regulation of hypersensitive cell death and disease defence by modulation of multiple signalling pathways, and provides important new information on the complex regulatory function of ERFs.

Keywords: cell death, defence, disease, ERF, tomato.

INTRODUCTION

Pathogen-induced plant immunity can be classified into two interconnected branches of responses: pathogen/microbe-associated molecular pattern (PAMP or MAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Cui *et al.*, 2015; Dangl *et al.*, 2013). PTI is triggered by the recognition of molecular signatures ubiquitously present in certain types of pathogen by cell surface-located receptors, i.e. pattern recognition receptors (PRRs). To fend off pathogens equipped with mechanisms to avoid and/or suppress PTI, host plants have evolved ETI that is mediated by R proteins via the direct or indirect perception of pathogen effectors. Following perception, PTI and ETI both involve the activation of certain common defence networks mediated by hormones, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), reactive oxygen species (ROS), kinases, transcriptional factors and the production of antimicrobial molecules and pathogenesis-related (PR) proteins (Baxter *et al.*, 2014; Cui *et al.*, 2015; Meng and Zhang, 2013; Pieterse *et al.*, 2012). However, temporal and quantitative regulation of these defence networks differs between PTI and ETI. In addition, ETI is often accompanied by programmed cell death (PCD), referred to as the hypersensitive response (HR), which occurs at the site of pathogen invasion and further restricts the spread of pathogens (Cui *et al.*, 2015).

The AP2 (APETALA2)/ERF (ethylene response factor) superfamily is the largest plant-specific transcription factor family and consists of diverse transcription factors. These proteins play crucial roles in plant signalling transduction pathways, switching extracellular signals into cellular responses, and are divided into families or groups based on the AP2/ERF domain (Licausi *et al.*, 2013; Muller and Munne-Bosch, 2015), including the ERF family. The ERF family can be further divided into 10 subgroups in *Arabidopsis*, 14 subgroups in rice and 11 subgroups in tomato (McGrath *et al.*, 2005; Nakano *et al.*, 2006; Sharma *et al.*, 2010). ERFs have important functions in plant responses to diseases and abiotic stresses (Licausi *et al.*, 2013; Mizoi *et al.*, 2012; Muller and Munne-Bosch, 2015). For example, some members of the ERF-IX subgroup, which contains activator-type ERFs, play important roles in plant disease responses (McGrath *et al.*, 2005; Nakano *et al.*, 2006). In tomato (*Solanum lycopersicum*), 18 ERFs have been identified as members of subgroup IX (Sharma *et al.*, 2010), and three have been characterized, including *TSRF1*, *Pti4* and *Pti5*. The expression of these three genes is regulated by defence-related phytohormones, PAMPs or pathogen infection (Zhang *et al.*, 2004; Zhou *et al.*, 1997), and transgenic plants overexpressing these ERFs confer enhanced tolerance to different pathogens, revealing their function in the positive regulation of disease defence (Gu *et al.*, 2002; He *et al.*, 2001; Zhang *et al.*, 2007). Furthermore, Pti4 and Pti5 participate in the tomato

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Pto/Prf-mediated ETI signalling pathway against avirulent *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. However, ERFs may play diverse roles in plant defence (Gu *et al.*, 2002) and most ERFs have not been characterized. In addition, despite the determined roles of a few tomato ERFs in disease responses, the involved signalling pathways and regulatory mechanisms are mostly not known. Gaining further insights into the function and regulatory mechanisms of these important proteins is of great significance.

PCD plays a crucial role in many plant functions, including disease defence responses (Dickman and Fluhr, 2013; Thomas, 2013). In addition to hypersensitive cell death, which occurs in incompatible plant–pathogen interactions, cell death also occurs in compatible plant–pathogen interactions and develops into disease symptoms, such as spots, specks or chlorosis. Furthermore, the expression patterns of genes involved in cell death at the early stage of incompatible interactions are similar to those at the late stage of compatible interactions, but at quantitatively different levels (Tao *et al.*, 2003). It is therefore very important for plants to precisely and distinctly control the expression of cell death. Accumulated studies have revealed important contributions of a few ERFs in the control of defence cell death. For instance, NbCD1 and NtERF3, two subgroup VIII repressor-type ERFs in tobacco, are involved in non-host resistance against *Pseudomonas cicholii* and *N* gene-mediated ETI resistance against *Tobacco mosaic virus*, respectively, by modulating cell death (Nasir *et al.*, 2005; Ogata *et al.*, 2013). In addition, MACD1, an ERF-IX member and a homologue of NtERF4 in *Nicotiana umbratica*, and its *Arabidopsis* homologue ERF102, participate in PCD triggered by phytotoxins produced by pathogens (Mase *et al.*, 2013). In tomato, Pti4/5/6 contribute to Pto/Prf-mediated ETI resistance by the activation of an array of *PR* genes (Gu *et al.*, 2002). However, additional regulators participating in defence cell death and the mediated regulatory mechanisms remain to be uncovered.

Our previous study revealed the involvement of tomato TSRF1, an ERF-IX member, in the defence response to bacterial wilt (BW) caused by *Ralstonia solanacearum* (*Rs*) (Chen *et al.*, 2009). In this study, we analysed the expression patterns of five uncharacterized tomato ERF-IX members after *Rs* infection, and selected the highly induced gene *ERF68* for further characterization. We show that ERF68 is a pathogen-induced positive regulator of hypersensitive cell death and disease defence by modulating multiple defence signalling pathways. Our study provides important new information on the complex regulatory mechanisms of plant ERFs.

RESULTS

Expression of tomato *ERF68* is induced by bacterial pathogens

To determine whether additional tomato ERF-IX members are involved in the BW defence response, we first searched for uncharacterized members that share sequence homology with TSRF1, Pti4 and Pti5 in the Sol genomic network (SGN) database. Five genes were selected: ERF56 (SGN-U566374; Solyc09g066360.1), ERF58 (SGN-U573501; Solyc04g014530.1), ERF67 (SGN-U572480; Solyc09g066360.1), ERF68 (SGN-U569393; Solyc08g078180.1) and ERF69 (SGN-U567854; Solyc01g 108740.2). These ERFs contain a typical ERF domain (Fig. S1a, see Supporting Information). On the basis of protein sequence homology, these proteins, together with other selected plant ERF-IX members with known functions, can be further divided into three subgroups (Fig. S1b).

Transcriptional analysis showed that, among the five selected uncharacterized ERF-IX members, *ERF68* expression in tomato was rapidly and dramatically induced after *Rs* inoculation (Fig. 1a). *ERF68* expression was also significantly induced by ET (Fig. 1b), SA (Fig. 1c) and pathogenic bacteria *Pst* DC3000 and *Xanthomonas euvesicatoria* (*Xeu*) Xvt28 (Fig. 1d). However, *ERF68* expression was only slightly induced after inoculation with type III secretion system (T3SS)-defective mutants of *Rs* and *Pst*, and the non-pathogenic bacterium *X. campestris* pv. *campestris* (*Xcc*) (Fig. 1d). These data reveal that *ERF68* is expressed on pathogen attack and suggest that ERF68 may be involved in pathways that are induced by bacterial T3SS effectors. In addition, *ERF68* was expressed more abundantly in roots and flower buds than in other tissues (Fig. S2, see Supporting Information).

Tomato ERF68 is a transcriptional activator and binds to the GCC-box

Subcellular localization assay showed that the ERF68-green fluorescent protein (ERF68-GFP) fusion was found in the nucleus (Fig. 2a). Because ERFs most probably bind to the GCC-box to exert their function in transcriptional regulation (Fujimoto *et al.*, 2000; Ohme-Takagi and Shinshi, 1995), two further assays were carried out to determine the regulatory effect of ERF68 on and binding affinity for the GCC-box. Transactivation assay showed that, similar to the known transcriptional activator *AtERF5*, *ERF68* expression significantly activated the expression of the GCC-box reporter (Fig. 2b), indicating that ERF68 acts as a functional transcriptional activator. Electrophoretic mobility shift assay (EMSA) was performed to further confirm the direct binding of ERF68 to the GCC-box. Because full-length ERF68 could not be produced in *Escherichia coli* despite various attempts using different protein expression systems, we prepared a glutathione transferase (GST) fusion protein containing the C-terminus (amino acids 123–183) of ERF68 instead. The resulting recombinant protein (GST-ERF68C), which contains the ERF domain, was used for EMSA. As shown in Fig. 2c, GST-ERF68C could bind to the GCC-box (AGCCGCC), and this binding was reduced by an unlabelled competitor probe, but not by a mutated competitor probe (AGAAGAA). Together, these results indicate that ERF68 can drive the

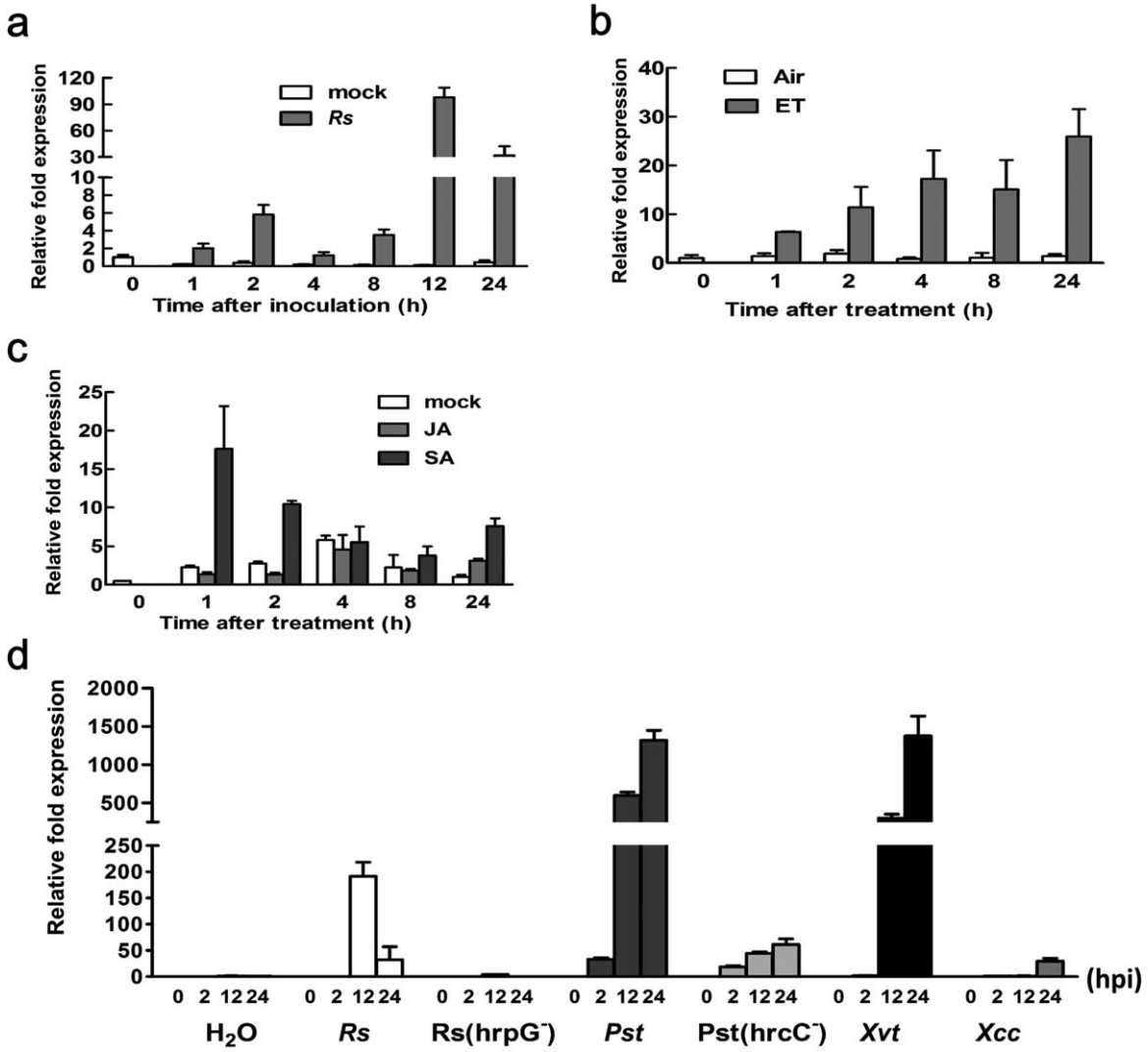

Fig. 1 Tomato *ERF68* is induced by pathogens and hormones. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to analyse *ERF68* expression in leaves of 3–4-week-old tomato H7996 after the indicated treatments. (a) Leaves vacuum infiltrated with *Ralstonia solanacearum* (*Rs*) Pss4 [10^7 colony-forming units (cfu)/mL]. (b) Plants treated with ethylene (ET) gas (10 ppm) or air in a sealed chamber. (c) Leaves sprayed with jasmonic acid (JA), salicylic acid (SA) or 0.01% ethanol (mock). (d) Leaves vacuum infiltrated with different bacterial pathogens (10^6 cfu/mL). *Pst*, *Pseudomonas syringae* pv. *tomato*; *Xcc*, *Xanthomonas campestris* pv. *campestris*; *Xvt*, *Xanthomonas euvesicatoria* (*Xeu*) Xvt28; hpi, h post-inoculation. The expression levels were normalized using tomato *ELONGATION FACTOR 1α* (*EF1α*) as the internal control. Values are means ± standard deviation. The data are from a single experiment that was repeated at least three times with similar results.

promoter activity of target genes via direct binding to specific *cis*-elements, such as the GCC-box.

Expression of tomato *ERF68* causes spontaneous cell death

To investigate the function of ERF68, virus-induced gene silencing (VIGS) and virus-mediated gene overexpression (VMGO) were used for further analyses. The results showed that transient systemic silencing of *ERF68* did not cause apparent phenotypic changes in tomato plants (Figs 3a and S3b, see Supporting Information); however, overexpression of *ERF68* led to severe growth defects (Fig. 3a) and spontaneous lesions on leaves (Figs 3b and S3c). These data indicate that ERF68 plays a role in the modulation of cell death in tomato. An inducible overexpression approach was further used in *Nicotiana benthamiana* to confirm the effect of *ERF68* overexpression on the triggering of cell death. The results showed that *EFR68* overexpression induced by β-estradiol treatment caused obvious cell death (Fig. 4a,b), which was further confirmed by increased cell conductivity (Fig.

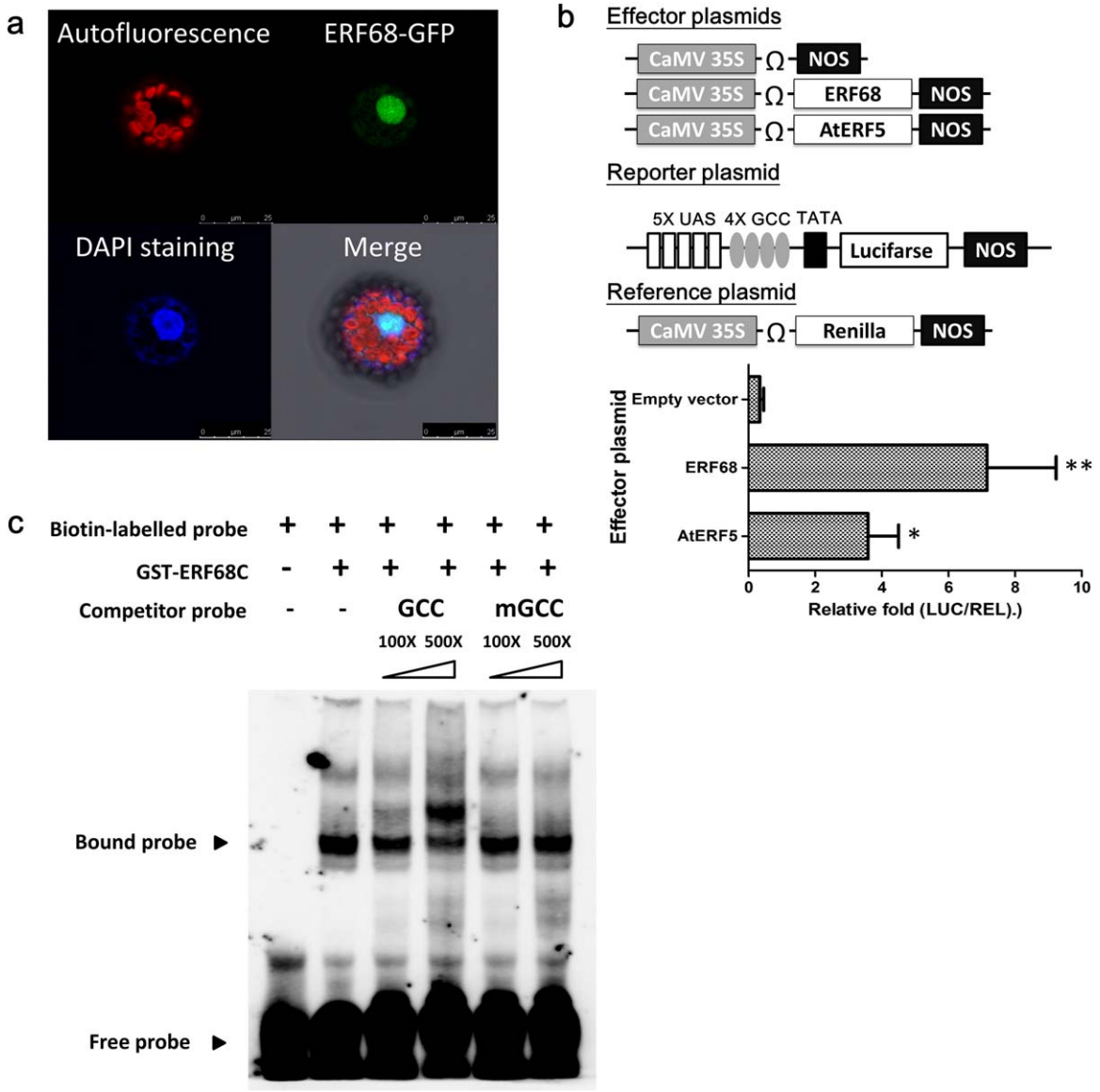

Fig. 2 ERF68 is localized in the nucleus and functions as a transcription factor. (a) Subcellular localization of ERF68. The vector carrying the ERF68-green fluorescent protein (ERF68-GFP) fusion was introduced into *Arabidopsis* protoplasts by polyethylene glycol (PEG) transfection. Photographs were taken at 16 h after transfection. 4′,6-Diamidino-2-phenylindole (DAPI) staining indicates the location of the nucleus. The experiment was repeated at least three times with similar results. (b) Transactivation assay of ERF68. Top panel: schematic diagram of constructions for transactivation assay. Bottom panel: activation of transcriptional activity of GCC-box-containing promoter by ERF68 and the positive control AtERF5. Total proteins were extracted from *Arabidopsis* protoplasts 16 h after co-transfection with reporter, reference and effector plasmids. 'Relative fold' indicates the relative fluorescence of firefly luciferase (LUC)/renilla luciferase (REL). For each independent experiment, three technical replicates were conducted. The data are from a single experiment that was repeated at least three times with similar results. Values are means ± standard deviation. Pair-wise comparisons between ERF68 or AtERF5 and vector control were made using Student's *t*-test. **Highly significant difference ($P < 0.01$). *Significant difference ($P < 0.05$). (c) Electrophoretic mobility shift assay (EMSA) for the physical interaction between a biotin-labelled probe containing the GCC-box and the C-terminal ERF (ethylene response factor) domain of ERF68 (GST-ERF68C). The experiment was repeated at least three times with similar results. GCC, unlabelled probe. mGCC, probe containing a mutated GCC box (AGAAGAA).

4c). These data indicate that ERF68 regulates plant cell death. However, the induction of *ERF68* overexpression did not alter the level of H_2O_2 accumulation (Fig. 3d), revealing that ERF68-mediated cell death is independent of the H_2O_2 burst. H_2O_2-independent cell death has also been described in another study (Lachaud *et al.*, 2011). However, the possible involvement of other ROS molecules in ERF68-mediated cell death cannot be excluded.

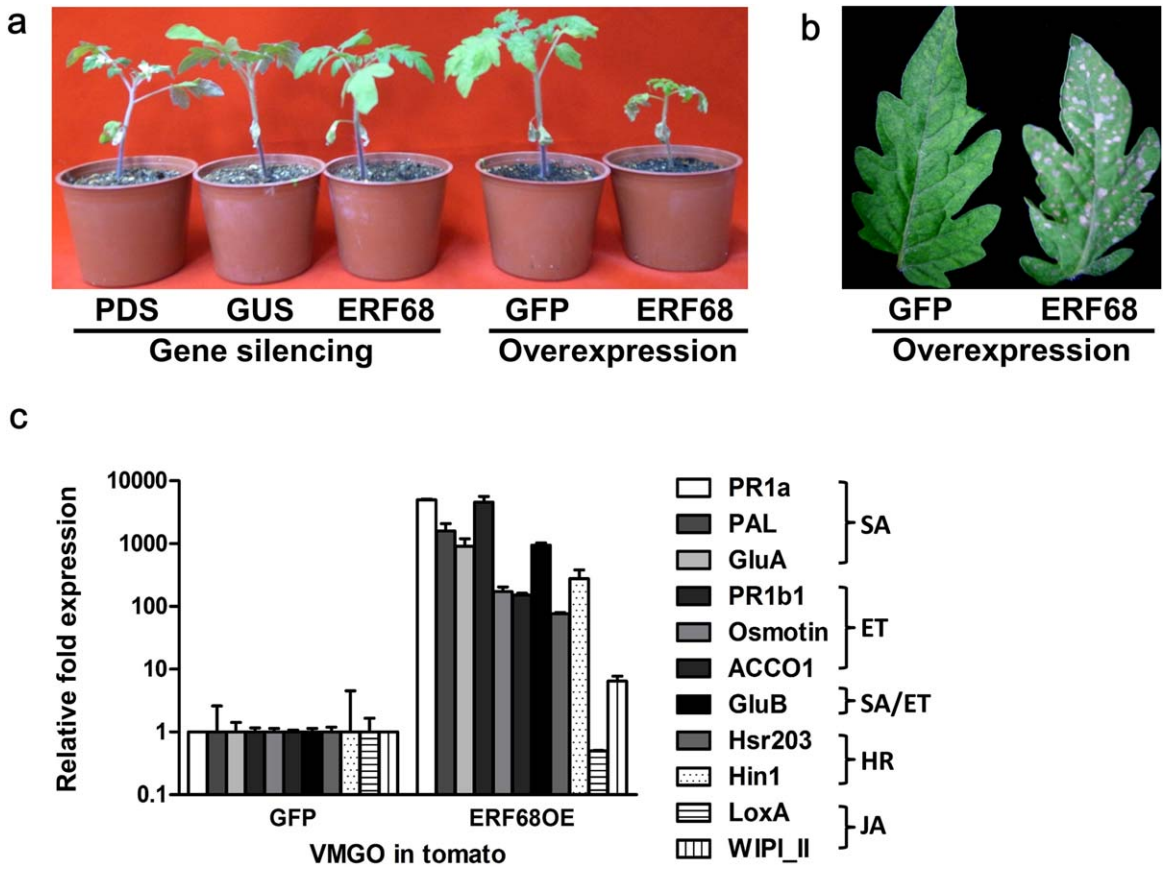

Fig. 3 Overexpression of *ERF68* causes spontaneous lesions and induces the expression of defence-related genes in tomato H7996. (a) Overall growth of the test plants. One-week-old plants were agroinfiltrated with the indicated virus-induced gene silencing (VIGS) or virus-mediated gene overexpression (VMGO) constructs at bacterial doses with an optical density at 600 nm (OD_{600}) of 2.0 and 0.8, respectively. Validation assays for *ERF68* expression were carried out in all experiments prior to further analyses to ensure the reliability of the test materials. The photograph was taken 2 weeks after agroinfiltration. (b, c) Ten-day-old plants were agroinfiltrated with the indicated VMGO constructs and the plants were used for further phenotypic and gene expression analyses 3 weeks after agroinfiltration. (b) Development of spontaneous lesions. (c) Expression of marker genes (see 'Results' for gene definitions) involved in defence pathways in leaves measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The samples are random combinations of at least four leaflets taken from at least two leaves of two tomato plants. The levels of expression are normalized using tomato *ELONGATION FACTOR 1α* (*EF1α*) as an internal control. The expression level of each gene in green fluorescent protein (GFP)-overexpressing plants is indicated as 1.0. Values are means ± standard deviation from three technical repeats in a single experiment that was repeated at least three times with similar results. ET, ethylene; GUS, β-glucuronidase; HR, hypersensitive response; JA, jasmonic acid; PDS, phytoene desaturase; SA, salicylic acid.

Expression of tomato *ERF68* enhances multiple defence signalling pathways

To learn more about the nature of ERF68-mediated cell death, we asked whether *ERF68* regulated disease defence signalling pathways. We assessed the effect of *ERF68* overexpression on the expression of well-known marker genes for tomato SA, ET, JA and HR signalling pathways. These included *PATHOGENESIS-RELATED 1a* (*PR1a*), *PHENYLALANINE AMMONIA LYASE* (*PAL*), *ENDO-GLUCANASE A* (*GluA*), *PATHOGENESIS-RELATED 1b1* (*PR1b1*), *OSMOTIN*, *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE 1* (*ACCO1*), *ENDO-GLUCANASE B* (*GluB*), *HYPERSENSITIVITY-RELATED GENE 203* (*Hsr203*), *HARPIN INDUCED PROTEIN 1* (*Hin1*), *LIPOXYGENASE A* (*LoxA*) and *WOUNDING INDUCED PROTEIN INHIBITOR II* (*WIPI-II*). As shown in Fig. 3d, the expression of marker genes for SA, ET and HR pathways was significantly induced in *ERF68*-overexpressing leaves, whereas *WIPI-II* expression was induced at a much lower level and *LoxA* expression remained unaltered. These data reveal the function of *ERF68* in the positive regulation of SA, ET, JA and HR signalling pathways.

Tomato *ERF68* plays a role in defence responses involved in specific pathosystems

Because *ERF68* regulates cell death (Figs 3 and 4) and is suggested to be involved in pathways induced by bacterial T3SS

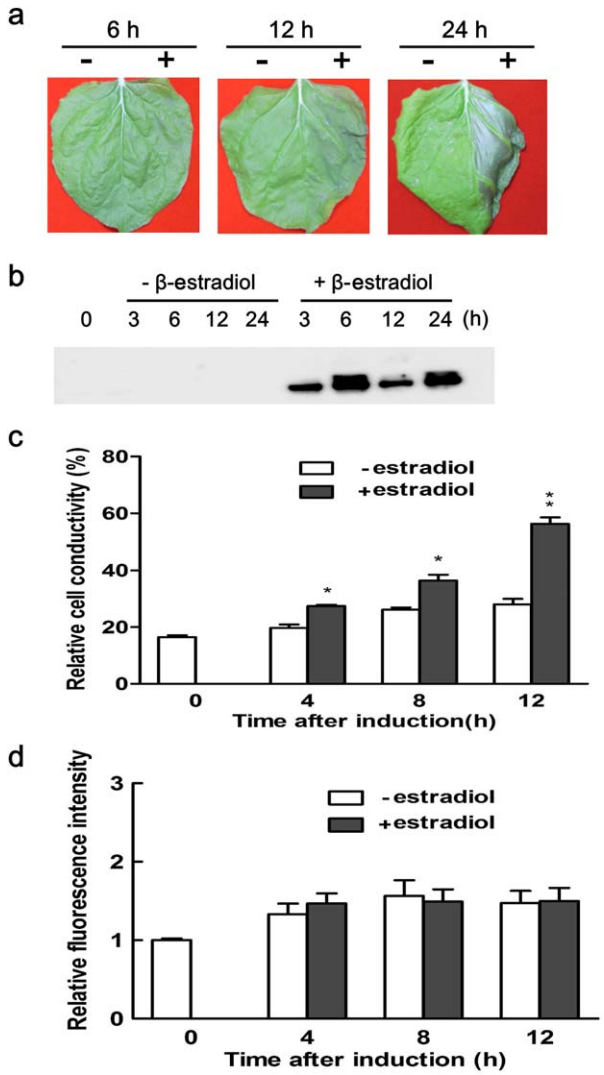

Fig. 4 Induced *ERF68* overexpression leads to cell death. Leaves of 4-week-old *Nicotiana benthamiana* were infiltrated with *Agrobacterium* carrying ERF68-HA driven by the β-estradiol inducible promoter. Two days after agroinfiltration, the leaves were infiltrated with β-estradiol (+ or + β-estradiol) or 0.005% ethanol (– or – β-estradiol). The samples were harvested at different time points after induction for the following analyses. (a) Development of cell death on leaf sections with induced ERF68-HA expression. (b) Detection of ERF68-HA by western blotting using a mouse anti-haemagglutinin (anti-HA) monoclonal antibody (H3663, Sigma-Aldrich). Fifty micrograms of each protein sample were used for western blotting. (c) Cell conductivity determined by the measurement of ion leakage. (d) H_2O_2 production detected by 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) staining. The accumulation of H_2O_2 was determined by relative fluorescence intensity with the level at 0 h as 1.0. (c, d) Values are means ± standard deviation. Pair-wise comparisons between β-estradiol-treated and ethanol-treated samples were made using Student's *t*-test. **Highly significant difference ($P < 0.01$). *Significant difference ($P < 0.05$). The data are from a single experiment that was repeated at least three times with similar results.

effectors (Fig. 1d), we investigated its role in tomato responses against different bacterial pathogens. First, we asked whether *ERF68* was involved in interactions between tomato and *Pst* DC3000. The resistant cultivar RG PtoR carrying the *R* locus *Pto-Prf* and the susceptible cultivar Moneymaker were used for *ERF68* VIGS assay. The results showed that silencing of *ERF68* did not cause notable changes in symptom severity (data not shown) or in *in planta* bacterial proliferation in both cultivars after inoculation with *Pst* DC3000 (Fig. S4, see Supporting Information), suggesting that ERF68 might not be involved in *Pto*-mediated ETI or in disease-associated cell death in these pathosystems.

Next, we examined whether *ERF68* was involved in the interactions between tomato and *Xanthomonas* spp. H7981 carries the *R* locus *XV3*, which confers resistance to *Xanthomonas* harbouring *avrXV3*, such as *Xeu* Xvt28; H7998 carries three non-dominant *R* genes, which confer resistance to *Xanthomonas* harbouring *avrRXV*, such as *X. perforans* Xtn50 (Stall *et al.*, 2009). Both cultivars confer compatible reactions with *X. vesicatoria* Xvt45. The results showed that silencing of *ERF68* caused increased bacterial proliferation in H7981 and H7998 after inoculation with incompatible *Xanthomonas* Xvt28 and Xtn50, respectively, but did not affect the compatible interactions (Fig. 5). These data indicate the involvement of *ERF68* in the tomato ETI response against *Xanthomonas*, but it has no effect on disease-associated cell death.

Furthermore, we checked whether *ERF68* was involved in the interactions between tomato and *Rs*. Silencing of *ERF68* did not cause significant changes in symptom development or proliferation of *Rs* in resistant cultivar H7996 (Fig. S5a, see Supporting Information), whose resistance is controlled by multiple quantitative trait loci (QTLs). Silencing of *ERF68* in susceptible cultivar L390 led to slightly more prompt symptom development (Fig. S5b), suggesting that ERF68 has a very minor effect, if any, on tomato defence against *Rs*.

Identification of ERF68 targets by chromatin immunoprecipitation combined with high-throughput sequencing (ChIP-seq)

To further investigate how ERF68 regulates defence cell death, we performed ChIP-seq to identify target genes regulated by ERF68. To precisely control *ERF68* overexpression and collect uniform high-quality samples, *N. benthamiana* leaves with β-estradiol-induced *ERF68* expression for 6 h were used for the assay. The sequencing results gathered from the immunoprecipitation (IP)-enrichment library were compared with the control library (input, without IP). The sequencing data are provided in Datasets S1–S3 (see Supporting Information). A total of 34 million trimmed reads (50 bp per read) was obtained from each of the IP and input libraries, in which 32 and 28 million reads were mapped to the *N. benthamiana* draft genome v0.4.4 scaffolds (Niben v0.4.4), respectively (Table S1, see Supporting Information). The read

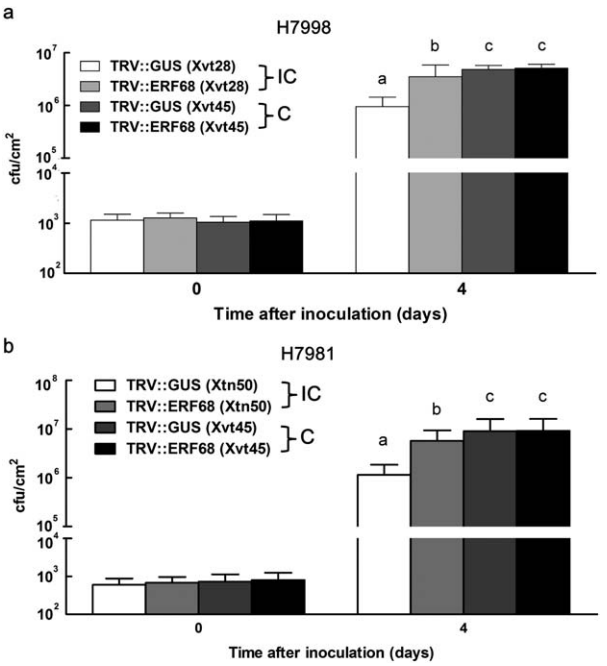

Fig. 5 Silencing of *ERF68* alters tomato defence responses to *Xanthomonas* spp. One-week-old tomato plants were agroinfiltrated with virus-induced gene silencing (VIGS) constructs. Three weeks after VIGS treatment, plants were vacuum infiltrated with the indicated avirulent and virulent *Xanthomonas* pathogens [optical density at 600 nm (OD_{600}) = 0.0004, 2×10^5 colony-forming units (cfu)/mL]. *In planta* proliferation of bacteria was determined. (a) Tomato cultivar H7998 was inoculated with *X. euvesicatoria* Xvt28 (incompatible, labelled as 'IC') and *X. vesicatoria* Xvt45 (compatible, labelled as 'C'). (b) Tomato cultivar H7981 was inoculated with *X. perforans* Xtn50 (incompatible, labelled as 'IC') and *X. vesicatoria* Xvt45 (compatible, labelled as 'C'). Values are means ± standard deviation from at least three independent experiments [$18 \times 4 = 72$ data for (a), $18 \times 3 = 54$ data for (b)]. Statistical analysis was performed using Duncan's test.

mapped rate was 93.62% for the IP library and 81.79% for the input library. The reads from the IP library were mapped to 2997 unique regions on Niben v0.4.4 scaffolds (Datasets S1–S3; Table S1).

To identify mapped regions that were located in genic regions of the *N. benthamiana* genome, we performed gene prediction to define possible genic locations. The range for gene prediction was 2000 bp upstream and downstream of the central position of the peak. The nature of the genic regions can be classified as: (1) promoter or transcriptional start site (TSS); (2) exon; (3) intergenic; and (4) no chromosome annotation because of gap interference or short scaffolds of the *N. benthamiana* genome draft. Among the 2997 unique mapping regions, 66 were predicted promoters or TSS, 80 were predicted exons, 761 were predicted intergenic regions and 2090 lacked chromosome annotation. Because ERFs most probably bind to the upstream regions of TSSs for their function in transcriptional regulation, we only focused on the 66 regions with predicted localization in the promoter or at the TSS. Amongst these 66 regions, only 31 regions were annotated as known or putative genes (Table S2, see Supporting Information). We further validated the ChIP-seq data by ChIP-qPCR on selected regions using two additional independent IP DNA samples prepared from leaves without or with β-estradiol-induced *ERF68* expression. The regions were chosen because of their involvement in defence or were randomly selected. The results showed that 10 of these regions were consistently significantly enriched in the *ERF68*-expressed IP samples compared with non-induced IP samples (Fig. S6, see Supporting Information; data not shown), confirming the reliability of our ChIP-seq results.

ERF68-dependent differential expression of target genes

Transcriptional analysis was carried out to determine whether ERF68 regulates the expression of the target genes. A BLAST search was performed to search for the cDNA sequence of each selected scaffold in the *N. benthamiana* genome v1.0.1 predicted cDNA database, and primers were designed for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of 28 annotated genes (Table S2). The results showed that the expression of five and three of these genes was consistently enhanced and repressed, respectively, by β-estradiol-induced *ERF68* expression (Fig. 6). Genes up-regulated by ERF68 included: *GLUTATHIONE S-TRANSFERASE* (*GSTU8*), *COATMER PROTEIN SUBUNIT α* (*COPA*), *ALLENE OXIDE SYNTHASE* (*AOS*), *TOSPOVIRUS RESISTANT PROTEIN 5a* (*Sw-5a*) and *OVATE FAMILY PROTEIN 2* (*OFP2*). Genes down-regulated by ERF68 included: *CHLOROPHYLL a/b BINDING PROTEIN* (*CAB*), *SOLUTE CARRIER FAMILY MEMBER 35 E3* (*SCF 35 E3*) and an unknown protein (Niben044Scf00001787).

DISCUSSION

Tomato ERF68 is a pathogen-induced regulator controlling multiple defence signalling pathways and cell death

ERFs are important regulators of various plant functions and play diverse roles. However, the functions and regulatory mechanisms of most tomato ERFs have not been determined. Previous studies have shown that tomato ERF-IX genes *Pti4*, *Pti5* and *TSRF1* are pathogen/PAMP induced and play a positive role in disease defence (Gu *et al.*, 2000; Nguyen *et al.*, 2010; Zhang *et al.*, 2004). Here, we show that tomato *ERF68* expression can be induced by pathogenic bacteria, with bacterial T3SS playing a major role (Fig. 1d), and that *ERF68* has a positive role in tomato defence against two incompatible *Xanthomonas* spp. (Fig. 5). These results point to its function in specific ETI pathways and add important new information on the versatile functions of tomato ERF-IX members

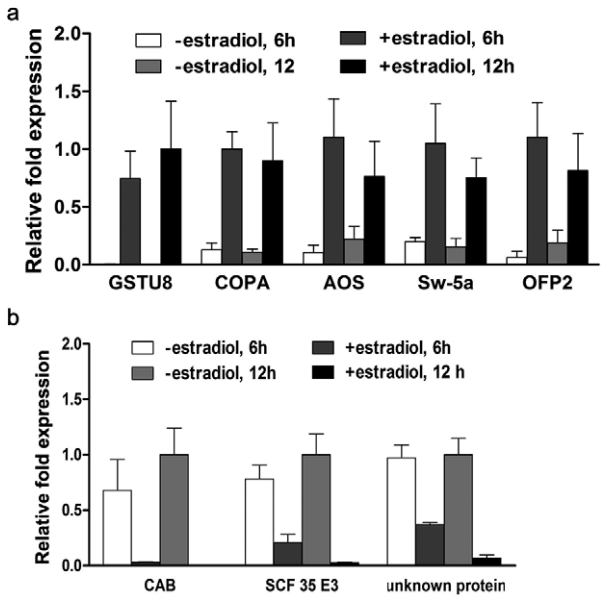

Fig. 6 ERF68 differentially regulates the target genes. Leaves of 4-week-old *Nicotiana benthamiana* were infiltrated with *Agrobacterium* carrying ERF68-HA driven by the β-estradiol inducible promoter. Two days after agroinfiltration, the leaves were infiltrated with β-estradiol (+ β-estradiol) or 0.01% ethanol (– β-estradiol). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to assess the expression level of selected target genes of ERF68. (a) Genes up-regulated by ERF68: *GSTU8*, *GLUTATHIONE S-TRANSFERASE*; *COPA*, *COATMER PROTEIN SUBUNIT α*; *AOS*, *ALLENE OXIDE SYNTHASE*; *Sw-5a*, *TOSPOVIRUS RESISTANT PROTEIN 5a*; *OFP2*, *OVATE FAMILY PROTEIN 2*. (b) Genes down-regulated by ERF68: *CAB*, *CHLOROPHYLL a/b BINDING PROTEIN*; *SCF 35 E3*, *SOLUTE CARRIER FAMILY MEMBER 35 E3*. The relative expression (fold change) was normalized using *Actin 1*. Values are means ± standard deviation from three technical repeats in a single experiment that was repeated at least three times with similar results.

in plant defence. In addition, *ERF68* expression can be induced by defence hormones SA and ET (Fig. 1b,c) and its overexpression significantly promotes the expression of several well-known marker genes involved in SA, ET and HR signalling (Fig. 3c), revealing its function in the positive regulation of these defence pathways. Because none of these ERF68-regulated genes was identified in our ChIP-seq results (Dataset S3; Table S2), ERF68 may modulate their expression indirectly by the regulation of additional regulators. In addition, *ERF68*, *Pti4* and *TSRF1* expression is induced by SA/ET and they play a positive role in the regulation of SA and ET pathways (Figs 1b,c and 3c; Gu *et al.*, 2002; Zhang *et al.*, 2004). However, interestingly, most ERF-IX members in *Arabidopsis*, including *ERF2* which is the homologue of *ERF68*, are regulated by JA/ET and participate in JA/ET signalling pathways and the related disease defence response (Lorenzo *et al.*, 2003; McGrath *et al.*, 2005; Moffat *et al.*, 2012; Onate-Sanchez and Singh, 2002; Onate-Sanchez *et al.*, 2007; Zhang *et al.*, 2015). Therefore, pathogen-induced ERF-IX members seem to play different regulatory roles in tomato and *Arabidopsis*.

Although the overexpression of a group of *Arabidopsis* and tomato ERF-IX genes leads to enhanced defence against different pathogens (Gu *et al.*, 2002; Lorenzo *et al.*, 2003; McGrath *et al.*, 2005; Moffat *et al.*, 2012; Onate-Sanchez and Singh, 2002; Onate-Sanchez *et al.*, 2007; Zhang *et al.*, 2004, 2015), the involvement of ERF-IX members in cell death has rarely been reported. Previously, Mase *et al.* (2013) have shown that *Nicotiana umbratica* ERF-IX gene *MACD1* and its *Arabidopsis* homologue *ERF102* are involved in PCD triggered by pathogen-produced phytotoxins via the regulation of ET and JA pathways, respectively (Mase *et al.*, 2013). However, the overexpression of these genes is insufficient to trigger PCD. In the current study, we have shown that *ERF68* overexpression is sufficient to cause spontaneous cell death in tomato and *N. benthamiana* (Figs 3 and 4), as well as to activate multiple defence pathways, including SA, ET, JA and HR pathways (Figs 3c and 6a). These results not only specify the novel function of ERF68 in the regulation of cell death, but also provide further insights into the distinct properties of ERF-IX members.

ERF68 differentially regulates its target genes and may bind to various *cis*-elements

Our data show that ERF68 acts as a transcriptional activator (Fig. 2b). However, interestingly, we found that ERF68 plays both positive and negative roles in the modulation of expression of its target genes (Fig. 6). Unlike ERF-VIII members, which act as transcriptional repressors and contain an EAR motif to recruit a co-repressor for repression activity (Ohta *et al.*, 2001), ERF-IX does not possess a known motif for its negative regulatory activity. However, we propose that, in addition to being a functional activator by itself, ERF68 can also function in the repression of target gene expression via interacting with other as yet undetermined repressor(s). Similarly, we also found that ERF68 binds the promoters or TSS of some target genes, but has no detectable effect on their expression. It is possible that overexpression of *ERF68* alone is insufficient to execute its regulatory function when the level of its co-regulator(s) is limited or in the absence of certain inducing factors. Our hypotheses are supported by the fact that heterodimers of some well-studied transcriptional factors are required for their regulatory activity. For example, potato Pti4 is required for the formation of a repressosome by recruiting repressor SEBF to repress *PR-10a* expression (Gonzalez-Lamothe *et al.*, 2008).

In addition, our data reveal that ERF68 binds to the GCC-box (Fig. 2c). Previous studies have shown that, in addition to the GCC-box, ERFs can also bind to other known and novel *cis*-elements (Cheng *et al.*, 2013; Sasaki *et al.*, 2007). We searched for predicted ERF68-binding *cis*-elements using the PlantCARE databases. Among the eight confirmed ERF68-regulated target genes (Fig. 6), the *COPA* promoter contains the *cis*-elements GCC-box,

DRE and HvCBF2, and the *Sw-5a* promoter contains DRE and HvCBF2 elements in both region-1 and region-4. These *cis*-elements are projected to be the ERF68-binding sites. In addition, we also attempted to search for known and putative novel ERF68-binding *cis*-elements on the 66 ChIP-identified regions with predicted localization on the promoter or at the TSS (Table S1). However, no consensus sequences with sufficient confidence were identified. Whether ERF68 can bind to additional as yet unidentified *cis*-elements remains to be investigated in the future, once a more comprehensive *N. benthamiana* genome sequence becomes available.

ERF68 regulates genes involved in hypersensitive cell death and disease defence

Among the seven target genes with annotated functions, the involvement of *GSTU8*, *OFP2* and *SCF 35 E3* in cell death or defence has not been reported. How ERF68 modulates plant cell death via the regulation of the four other target genes is discussed here.

The promoter of *COPA* contains the *cis*-elements GCC-box, DRE and HvCBF2. COPA is a member of the coatomer complex, which functions in the formation of the transport vesicle and is recruited by ADP-ribosylation factor (ARF1) to the site of vesicle budding in the Golgi apparatus (Popoff *et al.*, 2011). In addition to vesicle transportation, ARF1 and the coatomer complex are also involved in stress responses, in which *ARF1* is induced by elicitor and non-host pathogen treatment and is involved in the triggering of cell death in *N. benthamiana* (Coemans *et al.*, 2008). Because EFR68 positively regulates *COPA* expression, we propose that ERF68-mediated up-regulation of *COPA* would promote the formation of the coatomer complex, which is then recruited by ARF1, leading to enhanced cell death.

The promoter of *Sw-5a* contains DRE and HvCBF2 elements in both region-1 and region-4. Tomato *Sw-5* genes confer broad-spectrum resistance to *Tospovirus* species and share high homology with the aphid-resistance gene *Mi* and the *Pst*-resistance gene *Prf* (Brommonschenkel *et al.*, 2000). *Sw-5b* confers resistance to *Tomato spotted wilt virus*, whereas the function of *Sw-5a* is not clear, but it is hypothesized to be involved in disease resistance (Spassova *et al.*, 2001). Because EFR68 positively regulates *Sw-5a* expression, we hypothesize that ERF68-mediated activation of *Sw-5a* would somehow activate hypersensitive cell death associated with ETI.

Oxylipins are plant lipid peroxidation derivatives resulting from the action of the Lox enzyme. Oxylipins are not only precursors for the biosynthesis of defence hormone JA, but also participate in the triggering of cell death, particularly in the activation of HR associated with ETI (Montillet *et al.*, 2005; Raffaele *et al.*, 2008). *AOS* encodes allene oxide synthase, which catalyses 13-hydroperoxy octadecadienoic acid (13-HPOT) to allene oxide, a precursor of 12-oxo-phytodienoic acid (OPDA). 13-HPOT and OPDA are oxylipin compounds. In this study, we show that ERF68 positively regulates *AOS* expression. We postulate that ERF68-mediated enhancement of *AOS* expression results in a high level of oxylipin accumulation, leading to the activation of hypersensitive cell death. Meanwhile, the JA signalling pathway might also be induced, as revealed by moderate up-regulation of *WIPI2* expression (Fig. 3c).

CAB functions as a coordinator of antenna pigments in photosystems I and II (Jansson, 1994). In *Arabidopsis*, *CAB* expression is repressed in spontaneous cell death mutant *lesion initiation 1* (*len1*) or by treatment with elicitor-induced necrosis factor (INF) (Ishikawa *et al.*, 2003; Matsumura *et al.*, 2003). Repressed *CAB* expression results in chlorophyll degradation and the loss of photosystems. Because EFR68 negatively regulates *CAB* expression, we assume that ERF68-mediated repression of *CAB* would play a role in the development of cell death to restrict the spread of pathogens, aiding in the establishment of plant defence.

Tomato ERF68 participates in ETI against incompatible *Xanthomonas* spp.

In tomato, *Pto*-mediated ETI against *Pst* involves the activation of mitogen-activated protein kinase (MAPK) and SA signalling pathways (Oh and Martin, 2011). In tomato H7996, QTL-mediated BW resistance involves MAPK, SA and ET pathways, and HR (Chen *et al.*, 2009; Roux *et al.*, 2014). Interestingly, although ERF68 positively regulates SA, ET and HR signalling, it does not play a decisive role in these defence responses (Figs S4 and S5). However, our data reveal that ERF68 indeed participates in ETI of tomato H7981 and H7998 against two incompatible *Xanthomonas* spp. (Fig. 5). In these tomato cultivars, ETI involves the activation of MAPK signalling in responsive to the perception of *Xanthomonas* effectors (Melech-Bonfil and Sessa, 2010). However, how the signal is transduced to cause HR remains unclear. Although it remains to be determined whether ERF68 is regulated by the MAPK cascade, our data together invite us to propose that ERF68 may be a mediator linking the MAPK cascade and tomato ETI against *Xanthomonas* (Fig. S7, see Supporting Information). In our hypothesized model, *ERF68* expression is induced by SA/ET signalling after perception of the presence of *Xanthomonas* effectors (Fig. S7). By regulating SA/ET/HR defence signalling pathways and differentially modulating the expression of a group of genes (e.g. *COPA*, *Sw-5a*, *AOS*, *CAB*), ERF68 promotes hypersensitive cell death and the expression of defence genes, leading to incompatible interactions between tomato and *Xanthomonas* spp. Therefore, this study provides new critical insights into these incompatible interactions.

The precise regulation of PCD is crucial for many plant functions, including the establishment of disease resistance. However, information on PCD modulators and the regulatory mechanisms involved is far from sufficient. Here, we report that tomato ERF68

functions in the regulation of hypersensitive cell death associated with ETI by the modulation of multiple defence signalling pathways and the differential regulation of genes involved in cell death and disease defence. We consider that our study provides important new information on the complex roles of ERFs and offers critical insights into the regulatory functions of ERF68 in plant defence.

EXPERIMENTAL PROCEDURES

Plant materials, bacterial strains and growth conditions

Tomato (*Solanum lycopersicum*) cultivars Hawaii 7981 (H7981), Hawaii 7996 (H7996), Hawaii 7998 (H7998), L390, Moneymaker and Rio Grande (RG) PtoR were used for transient gene silencing and overexpression experiments (Moneymaker and RG PtoR seeds were kindly provided by Dr Lin Nai-Chun, Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan, China). *Nicotiana benthamiana* was used for transient protein expression and ChIP-seq. *Arabidopsis thaliana* Col0 was used for the preparation of protoplasts. Tomato and *N. bemthamiana* were grown in growth chambers at 25 °C under a 12-h light/12-h dark cycle. *Arabidopsis* was grown in growth chambers at 21 °C under an 8-h light/16-h dark cycle. The bacteria used in this study included: *Escherichia coli* DH5α, *Agrobacterium tumefaciens* GV3101 and MOG101, *Pst* DC3000 and its T3SS-defective mutant *hrcC*$^-$ (kindly provided by Laurent Zimmerli, Institute of Plant Biology, National Taiwan University, Taipei, Taiwan, China), *Rs* Pss4 (phylotype I, biovar 3) and its T3SS-defective mutant *hrpG*$^-$, *Xeu* Xvt28, *X. perforans* Xtn50, *X. vesicatoria* Xvt45 and *Xcc*. *Escherichia coli* was grown in Luria–Bertani (LB) medium; *Ag. tumefaciens* was grown in yeast extract-peptone (YEP) medium; *Rs* and *Xanthomonas* spp. were grown in 523 medium; *Pst* DC3000 was grown in King's broth. *Escherichia coli* was grown at 37 °C, and all other bacteria were grown at 28 °C.

Cloning and construction of tomato *ERF68*

The full length or fragments of *ERF68* were amplified from cDNA of tomato H7996 by PCR. The PCR products were cloned into pCR®8/GW/TOPO® (pCR8, Invitrogen, Carlsbad, CA, USA), and then introduced into different vectors for the indicated analyses as described below. All primers used in this study are listed in Table S3 (see Supporting Information).

Treatments and transcriptional analyses for tomato *ERF68* expression

Three- to four-week-old tomato H7996 plants were used for treatments with hormones or pathogens. For hormone treatments, plants were treated with ET gas (10 ppm) or air in a sealed chamber, or sprayed with JA (0.1 μM), SA (2 μM) or ethanol (0.01%). Pathogens used to inoculate plants were grown in appropriate liquid medium overnight at 28 °C. The bacterial cultures were collected by centrifugation, resuspended in $MgCl_2$ (10 mM) and adjusted to 10^6 colony-forming units (cfu)/mL [optical density at 600 nm (OD_{600}) of approximately 0.002 or 0.003] for vacuum inoculation on tomato leaves. Leaf samples were then harvested at the indicated time points for RT-qPCR or semi-quantitative RT-PCR (RT-sqPCR) assays, as described previously (Lin *et al*., 2014). The samples were randomly combined from at least four leaflets taken from two leaves of two tomato plants. Tomato *ELONGATION FACTOR 1α* (*EF1α*) and *Ubiquitin 3* (*Ubi3*) and *N. benthamiana Actin 1*, whose expression levels were not modulated by hormone treatments or pathogen infection, or altered expression of tomato *ERF68*, were used as internal controls to normalize gene expression. All experiments were repeated at least thrice at different times using different samples. All primers are listed in Table S3.

Protein localization and transactivation assay

Protoplasts were prepared from 4-week-old *A. thaliana* Col0 plants as described previously (Lee *et al*., 2012). For protein localization assay, the full length of *ERF68* without the stop codon was cloned into p2FGW7,0 for ERF68-GFP expression, and 20 μg of the construct were used. The location of the plant nucleus was indicated by treatment with DAPI (4′,6-diamidino-2-phenylindole, 4 μM) for 30 min before examination. For the transactivation assay, full-length *ERF68* was cloned into an effector plasmid, a kind gift from Dr Masaru Ohme-Takagi [Institute for Environmental Science and Technology (IEST), Saitama University, Saitama, Japan] and used for analysis (20 μg; effector/reporter/reference plasmids = 4 : 5 : 1). The relative luciferase activity indicates the average ratio of firefly luciferase luminescence and renilla luciferase luminescence. All experiments were repeated at least thrice at different times using different samples.

Electrophoretic mobility shift assay (EMSA)

To prepare GST fusion protein for EMSA, the C-terminus (amino acids 123–183) of tomato *ERF68* (ERF68C), which contains the ERF domain, was amplified by PCR, digested with *Bam*HI and *Xho*I, and cloned into pGEX 4T-1. The resulting construct was introduced into *E. coli* BL21DE3 for GST-ERF68C expression. After induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h, the recombinant protein was purified by Glutathione Sepharose™ (GE Healthcare, SE-751 84 Uppsala, Sweden). Biotin-labelled, non-labelled and mutated probes were synthesized by Mission Biotech Company (New Taipei City, Taiwan) (Table S3). Probe annealing and EMSA were carried out following the instructions described in 'Tech Tip: Anneal complementary pairs of oligonucleotides' and the LightShift™ Chemiluminescence EMSA kit manual (Thermo Fisher Scientific, Rockford, IL, USA). GST-ERF68C (1 μM) was mixed with the biotin-labelled probes (20 nM) for the experiments. The unlabelled probes (100×, 2 μM; 500×, 10 μM) were used for competition tests.

VIGS and VMGO

One-week-old tomato plants were used for the assays. For *Tobacco rattle virus* (TRV)-based VIGS, preparation of gene-specific TRV-VIGS constructs and VIGS assays in tomato were carried out as described previously (Chen *et al*., 2009). Different doses of a mixture of *Ag. tumefaciens* GV3101 carrying *TRV1* and different *TRV2* constructs (1 : 1 ratio) were used for infiltration on cotyledons: H7996 (OD_{600} = 2.0), L390 (OD_{600} = 2.0), Moneymaker (OD_{600} = 2.0), RG PtoR (OD_{600} = 2.0), H7981 (OD_{600} = 0.15), H7998 (OD_{600} = 0.15). *TRV*::*GUS* was used as a negative control. For *Potato virus X* (PVX)-based VMGO, full-length *ERF68* or *GFP* was cloned into the *Cla*I-*Not*I site of vector pSfinX and then introduced into *Ag. tumefaciens* MOG101. Bacterial overnight cultures grown in YEP

broth were collected by centrifugation and resuspended in inoculation buffer [10 mM $MgCl_2$, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 200 μM acetosyringone, pH 5.6], followed by incubation for 4–6 h at 28 °C to induce the virulence of *Agrobacterium*. Cotyledons were infiltrated with *Agrobacterium* (OD_{600} = 0.8) carrying the indicated VMGO constructs. The VIGS and VMGO plants were grown at 21 °C for 2–3 weeks. The efficiency of *ERF68* silencing and overexpression was confirmed in all experiments by carrying out RT-sqPCR or RT-qPCR (Fig. S3a) prior to further analyses to ensure the reliability of the test materials. Tomato *EF1α* and *Ubi3*, whose expression levels were not modulated by altered expression of tomato *ERF68*, were used as internal controls to normalize gene expression. All experiments were repeated at least thrice at different times using different samples.

Induced protein expression and analyses of cell conductivity and H_2O_2 accumulation

Full-length *ERF68* fused with C-terminal 3 × haemagglutinin (HA) and the *NOS* (*nopaline synthase*) terminator was cloned into the *Xho*I-*Spe*I site of the β-estradiol inducible vector pMDC7 (Curtis & Grossniklaus, 2003), yielding pMDC-ERF68-cHA. To induce *in planta* protein expression, *Agrobacterium* GV3101 (OD_{600} = 0.8) carrying pMDC-ERF68-cHA was infiltrated into *N. benthamiana* leaves. After 48 h, β-estradiol (40 μM) or control reagent (0.005% ethanol) was infiltrated again into the infiltrated leaves to induce ERF68-cHA production. The treated leaves were harvested at various time points for further analyses, including phenotyping, western blotting, cell conductivity and detection of H_2O_2 accumulation. For analyses of cell conductivity and H_2O_2 accumulation, the methods described by Nasir *et al.* (2005) were followed. For the cell conductivity test, each technical replicate contained two discs (1 cm in diameter) taken from each leaf, and data were collected from 12 technical replicates for each independent experiment. For H_2O_2 detection, 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) staining was used to monitor H_2O_2 content in agroinfiltrated leaves after β-estradiol induction. DCFDA (10 μM) was infiltrated into pretreated leaves for 30 min. Ten leaf discs were taken from 10 treated leaves used to measure the relative fluorescence values. All experiments were repeated at least thrice at different times using different samples.

Assessment of plant disease responses

Plant responses to infection by *Pst* DC3000 and *Xanthomonas* spp. were assessed following the methods described by Melech-Bonfil and Sessa (2010) with modifications. Briefly, *Pst* DC3000 and *Xanthomonas* spp. were grown overnight in King's broth and 523 liquid medium, respectively. The bacterial cultures were collected, resuspended and diluted in $MgCl_2$ (10 mM, with 0.005% Silwet-77) to give the indicated concentrations, and used for plant inoculation by vacuum infiltration. *In planta Xanthomonas* proliferation was monitored at the indicated time points after inoculation. Each technical replicate, which contained four leaf discs (8 mm in diameter) taken from four different leaflets randomly collected from four plants, was ground, serially diluted and spotted onto two duplicate medium plates containing appropriate antibiotics. For each independent experiment, eight plants were used to collect leaf discs for nine technical replicates. The assessments of bacterial wilting caused by *Rs* and bacterial proliferation were carried out as described previously (Chen *et al.*, 2009). All experiments were repeated at least thrice at different times using different samples.

ChIP-seq and ChIP-qPCR

The ChIP-seq methods described by Kaufmann *et al.* (2010) and Zhu *et al.* (2012) were followed with modifications. Briefly, *N. benthamiana* leaves (10 g) with β-estradiol-induced ERF68-cHA expression were harvested at 6 h after induction. The leaf samples were fixed with 0.1% formaldehyde by vacuum infiltration for 1 min and quenched with glycine (0.6 M) for 5 min. The leaves were then rinsed with double-distilled H_2O several times, dried on paper towels and ground in liquid nitrogen. Chromatin complexes were isolated, homogenized with a sonicator and then incubated with a mouse anti-HA monoclonal antibody (H3663, Sigma-Aldrich, St. Louis, MO, USA). The immunoprecipitated DNA and crude chromatin complexes (without IP) were recovered and subjected to high-throughput sequencing or qPCR. At least 15 ng of the DNA fragments, which were collected and combined from three independent experiments, were used for high-throughput sequencing. Library generation, Illumina next generation sequencing (NGS) and preliminary bioinformatics analyses were conducted by Yourgene Bioscience Company (Shulin, New Taipei City, Taiwan).

The trimmed reads were aligned to the *N. benthamiana* draft genome v0.4.4 (SOL Genomics Network database; Bombarely *et al.*, 2012) using Burrows–Wheeler Alignment (BWA) (Li and Durbin, 2009). Redundant reads were removed from the mapping results by SAMtools (Sequence Alignment Map format). This process was expected to yield more reliable peak calls for further analyses. The identification of genome-wide locations of transcription factor binding from ChIP-seq was calculated by the Model-based analysis of ChIP-seq (MACS) algorithm, which consists of four steps: removal of redundant reads, adjustment of read position, calculation of peak enrichment and estimation of the empirical false discovery rate (FDR). By sample swap, MACS uses the same parameters to find ChIP peaks over control and control peaks over ChIP to examine the *P* values of the peaks. The information and statistics of peak detection are provided in Datasets S1–S3. The locations of the peaks and summits were viewed using the IGV genome browser. Neighbour genes were defined as genes located approximately 2000 bp upstream and downstream from the centre of each peak. The neighbour genes of each peak were identified using HOMER script (Glass Lab, University of California, San Diego, CA, USA) and the annotation file downloaded from the SOL Genomics Network database. The same method was used to identify the nearest promoters or TSSs to each peak.

For ChIP-qPCR, immunoprecipitated DNA samples collected from leaves with or without β-estradiol-induced ERF68-cHA expression were used for qPCR with specific primers (Table S3). Values obtained from the precipitated DNA were normalized with their corresponding crude chromatin complexes. The experiment was repeated at least thrice at different times using different samples.

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REFERENCES

Baxter, A., Mittler, R. and Suzuki, N. (2014) ROS as key players in plant stress signalling. *J. Exp. Bot.* **65**, 1229–1240.

Bombarely, A., Rosli, H.G., Vrebalov, J., Moffett, P., Mueller, L.A. and Martin, G.B. (2012) A draft genome sequence of *Nicotiana benthamiana* to enhance molecular plant–microbe biology research. *Mol. Plant–Microbe Interact.* **25**, 1523–1530.

Brommonschenkel, S.H., Frary, A., Frary, A. and Tanksley, S.D. (2000) The broad-spectrum tospovirus resistance gene *Sw-5* of tomato is a homolog of the root-knot nematode resistance gene *Mi. Mol. Plant–Microbe Interact.* **13**, 1130–1138.

Chen, Y.Y., Lin, Y.M., Chao, T.C., Wang, J.F., Liu, A.C., Ho, F.I. and Cheng, C.P. (2009) Virus-induced gene silencing reveals the involvement of ethylene-, salicylic acid- and mitogen-activated protein kinase-related defense pathways in the resistance of tomato to bacterial wilt. *Physiol. Plant.* **136**, 324–335.

Cheng, M.C., Liao, P.M., Kuo, W.W. and Lin, T.P. (2013) The Arabidopsis ETHYLENE RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to different *cis*-acting elements in response to different stress signals. *Plant Physiol.* **162**, 1566–1582.

Coemans, B., Takahashi, Y., Berberich, T., Ito, A., Kanzaki, H., Matsumura, H., Saitoh, H., Tsuda, S., Kamoun, S., Sági, L., Swennen, R. and Terauchi, R. (2008) High-throughput *in planta* expression screening identifies an ADP-ribosylation factor (*ARF1*) involved in non-host resistance and *R* gene-mediated resistance. *Mol. Plant Pathol.* **9**, 25–36.

Cui, H., Tsuda, K. and Parker, J.E. (2015) Effector-triggered immunity: from pathogen perception to robust defense. *Annu. Rev. Plant Biol.* **66**, 487–511.

Curtis, M.K. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiol.* **133**, 462–469.

Dangl, J.L., Horvath, D.M. and Staskawicz, B.J. (2013) Pivoting the plant immune system from dissection to deployment. *Science*, **341**, 746–751.

Dickman, M.B. and Fluhr, R. (2013) Centrality of host cell death in plant–microbe interactions. *Annu. Rev. Phytopathol.* **51**, 543–570.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell*, **12**, 393–404.

Gonzalez-Lamothe, R., Boyle, P., Dulude, A., Roy, V., Lezin-Doumbou, C., Kaur, G.S., Bouarab, K., Després, C. and Brisson, N. (2008) The transcriptional activator Pti4 is required for the recruitment of a repressosome nucleated by repressor SEBF at the potato *PR-10a* gene. *Plant Cell*, **20**, 3136–3147.

Gu, Y.Q., Yang, C., Thara, V.K., Zhou, J. and Martin, G.B. (2000) *Pti4* is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. *Plant Cell*, **12**, 771–785.

Gu, Y.Q., Wildermuth, M.C., Chakravarthy, S., Loh, Y.T., Yang, C., He, X. and Martin, G.B. (2002) Tomato transcription factors Pti4, Pti5, and Pti6 activate defense responses when expressed in Arabidopsis. *Plant Cell*, **14**, 817–831.

He, P., Warren, R.F., Zhao, T., Shan, L., Zhu, L., Tang, X. and Zhou, J.M. (2001) Overexpression of *Pti5* in tomato potentiates pathogen-induced defense gene expression and enhances disease resistance to *Pseudomonas syringae* pv. *tomato*. *Mol. Plant–Microbe Interact.* **14**, 1453–1457.

Ishikawa, A., Tanaka, H., Nakai, M. and Asahi, T. (2003) Deletion of a chaperonin 60 beta gene leads to cell death in the *Arabidopsis lesion initiation* 1 mutant. *Plant Cell Physiol.* **44**, 255–261.

Jansson, S. (1994) The light-harvesting chlorophyll a/b-binding proteins. *Biochim. Biophys. Acta*, **1184**, 1–19.

Kaufmann, K., Muiño, J.M., Østerås, M., Farinelli, L., Krajewski, P. and Angenent, G.C. (2010) Chromatin immunoprecipitation (ChIP) of plant transcription factors followed by sequencing (ChIP-SEQ) or hybridization to whole genome arrays (ChIP-CHIP). *Nat. Protoc.* **5**, 457–472.

Lachaud, C., Prigent, E., Thuleau, P., Grat, S., Da Silva, D., Briére, C., Mazars, C. and Cotelle, V. (2013) 14-3-3-regulated Ca^{2+}-dependent protein kinase CPK3 is required for sphingolipid-induced cell death in Arabidopsis. *Cell Death Differ.* **20**, 209–217.

Lee, L.Y., Wu, F.H., Hsu, C.T., Shen, S.C., Yeh, H.Y., Liao, D.C., Fang, M.J., Liu, N.T., Yen, Y.C., Dokládal, L., Sýkorová, E., Gelvin, S.B. and Lin, C.S. (2012) Screening a cDNA library for protein-protein interactions directly in *Planta. Plant Cell* **24**, 1746–1759.

Li, H. and Durbin, R. (2009) Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics*, **25**, 1754–1760.

Licausi, F., Ohme-Takagi, M. and Perata, P. (2013) APETALA2/ethylene responsive factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. *New Phytol.* **199**, 639–649.

Lin, Y.M., Shih, S.L., Lin, W.C., Wu, J.W., Chen, Y.T., Hsieh, C.Y., Guan, L.C., Lin, L. and Cheng, C.P. (2014) Phytoalexin biosynthesis genes are regulated and involved in plant response to *Ralstonia solanacearum* infection. *Plant Sci.* **224**, 86–94.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J. and Solano, R. (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell*, **15**, 165–178.

Mase, K., Ishihama, N., Mori, H., Takahashi, H., Kaminaka, H., Kodama, M. and Yoshioka, H. (2013) Ethylene-responsive AP2/ERF transcription factor MACD1 participates in phytotoxin-triggered programmed cell death. *Mol. Plant–Microbe Interact.* **26**, 868–879.

Matsumura, H., Reich, S., Ito, A., Saitoh, H., Kamoun, S., Winter, P., Kahl, G., Reuter, M., Kruger, D.H. and Terauchi, R. (2003) Gene expression analysis of plant host–pathogen interactions by SuperSAGE. *Proc. Natl. Acad. Sci. USA*, **100**, 15 718–15 723.

McGrath, K.C., Dombrecht, B., Manners, J.M., Schenk, P.M., Edgar, C.I., Maclean, D.J., Scheible, W.R., Udvardi, M.K. and Kazan, K. (2005) Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. *Plant Physiol.* **139**, 949–959.

Melech-Bonfil, S. and Sessa, G. (2010) Tomato MAPKKKε is a positive regulator of cell-death signaling networks associated with plant immunity. *Plant J.* **64**, 379–391.

Meng, X. and Zhang, S. (2013) MAPK cascades in plant disease resistance signaling. *Annu. Rev. Phytopathol.* **51**, 245–266.

Mizoi, J., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2012) AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta*, **1819**, 86–96.

Moffat, C.S., Ingle, R.A., Wathugala, D.L., Saunders, N.J., Knight, H. and Knight, M.R. (2012) ERF5 and ERF6 play redundant roles as positive regulators of JA/Et-mediated defense against *Botrytis cinerea* in Arabidopsis. *PLoS One*, **7**, e35995.

Montillet, J.L., Chamnongpol, S., Rusterucci, C., Dat, J., van de Cotte, B., Agnel, J.P., Battesti, C., Inzé, D., Van Breusegem, F. and Triantaphylidès, C. (2005) Fatty acid hydroperoxides and H_2O_2 in the execution of hypersensitive cell death in tobacco leaves. *Plant Physiol.* **138**, 1516–1526.

Muller, M. and Munne-Bosch, S. (2015) Ethylene response factors: a key regulatory hub in hormone and stress signaling. *Plant Physiol.* **169**, 32–41.

Nakano, T., Suzuki, K., Fujimura, T. and Shinshi, H. (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiol.* **140**, 411–432.

Nasir, K.H., Takahashi, Y., Ito, A., Saitoh, H., Matsumura, H., Kanzaki, H., Shimizu, T., Ito, M., Fujisawa, S., Sharma, P.C., Ohme-Takagi, M., Kamoun, S. and Terauchi, R. (2005) High-throughput in planta expression screening identifies a class II ethylene-responsive element binding factor-like protein that regulates plant cell death and non-host resistance. *Plant J.* **43**, 491–505.

Nguyen, H.P., Chakravarthy, S., Velasquez, A.C., McLane, H.L., Zeng, L., Nakayashiki, H., Park, D.H., Collmer, A. and Martin, G.B. (2010) Methods to study PAMP-triggered immunity using tomato and *Nicotiana benthamiana*. *Mol. Plant–Microbe Interact.* **23**, 991–999.

Ogata, T., Kida, Y., Tochigi, M. and Matsushita, Y. (2013) Analysis of the cell death-inducing ability of the ethylene response factors in group VIII of the AP2/ERF family. *Plant Sci.* **209**, 12–23.

Oh, C.S. and Martin, G.B. (2011) Effector-triggered immunity mediated by Pto kinase. *Trends Plant Sci.* **16**, 132–140.

Ohme-Takagi, M. and Shinshi, H. (1995) Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell*, **7**, 173–118.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H. and Ohme-Takagi, M. (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell*, **13**, 1959–1968.

Onate-Sanchez, L. and Singh, K.B. (2002) Identification of Arabidopsis ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection. *Plant Physiol.* **128**, 1313–1322.

Onate-Sanchez, L., Anderson, J.P., Young, J. and Singh, K.B. (2007) AtERF14, a member of the ERF family of transcription factors, plays a nonredundant role in plant defense. *Plant Physiol.* **143**, 400–409.

Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S.C. (2012) Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* **28**, 489–521.

Popoff, V., Adolf, F., Brugger, B. and Wieland, F. (2011) COPI budding within the Golgi stack. *Cold Spring Harbor Persp. Biol.* **3**, a005231.

Raffaele, S., Vailleau, F., Leger, A., Joubes, J., Miersch, O., Huard, C., Blée, E., Mongrand, S., Domergue, F. and Roby, D. (2008) A MYB transcription factor regulates very-long-chain fatty acid biosynthesis for activation of the hypersensitive cell death response in Arabidopsis. *Plant Cell*, **20**, 752–767.

Roux, F., Voisin, D., Badet, T., Balague, C., Barlet, X., Huard-Chauveau, C., Roby, D. and Raffaele, S. (2014) Resistance to phytopathogens e tutti quanti: placing plant quantitative disease resistance on the map. *Mol. Plant Pathol.* **15**, 427–432.

Sasaki, K., Mitsuhara, I., Seo, S., Ito, H., Matsui, H. and Ohashi, Y. (2007) Two novel AP2/ERF domain proteins interact with *cis*-element VWRE for wound-induced expression of the tobacco *tpoxN1* gene. *Plant J.* **50**, 1079–1092.

Sharma, M.K., Kumar, R., Solanke, A.U., Sharma, R., Tyagi, A.K. and Sharma, A.K. (2010) Identification, phylogeny, and transcript profiling of ERF family genes during development and abiotic stress treatments in tomato. *Mol. Genet. Genomics*, **284**, 455–475.

Spassova, M.I., Prins, W., Folkertsma, R.T., Klein-Lankhorst, R.M., Hille, J., Goldbach, R.W. (2001) The tomato gene *Sw5* is a member of the coiled coil, nucleotide binding, leucine-rich repeat class of plant resistance genes and confers resistance to TSWV in tobacco. *Mol. Breed.* **7**, 151–161.

Stall, R.E., Jones, J.B. and Minsavage, G.V. (2009) Durability of resistance in tomato and pepper to xanthomonads causing bacterial spot. *Annu. Rev. Phytopathol.* **47**, 265–284.

Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G. and Katagiri, F. (2003) Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. *Plant Cell*, **15**, 317–330.

Thomas, H. (2013) Senescence, ageing and death of the whole plant. *New Phytol.* **197**, 696–711.

Zhang, H., Zhang, D., Chen, J., Yang, Y., Huang, Z., Huang, D., Wang, X.C. and Huang, R. (2004) Tomato stress-responsive factor TSRF1 interacts with ethylene responsive element GCC box and regulates pathogen resistance to *Ralstonia solanacearum*. *Plant Mol. Biol.* **55**, 825–834.

Zhang, H., Li, W., Chen, J., Yang, Y., Zhang, Z., Zhang, H., Wang, X.C. and Huang, R. (2007) Transcriptional activator TSRF1 reversely regulates pathogen resistance and osmotic stress tolerance in tobacco. *Plant Mol. Biol.* **63**, 63–71.

Zhang, H., Huang, L., Dai, Y., Liu, S., Hong, Y., Tian, L., Huang, L., Cao, Z, Li, D. and Song, F. (2015) Arabidopsis AtERF15 positively regulates immunity against *Pseudomonas syringae* pv. *tomato* DC3000 and *Botrytis cinerea*. *Front. Plant Sci.* **6**, 686.

Zhou, J., Tang, X. and Martin, G.B. (1997) The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of *pathogenesis-related* genes. *EMBO J.* **16**, 3207–3218.

Zhu, J.Y., Sun, Y. and Wang, Z.Y. (2012) Genome-wide identification of transcription factor-binding sites in plants using chromatin immunoprecipitation followed by microarray (ChIP-chip) or sequencing (ChIP-seq). *Methods Mol. Biol.* **876**, 173–188.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Protein alignment and phylogenetic analysis of selected members of ethylene response factor (ERF) group IX.

Fig. S2 Tomato ERF68 is differentially expressed in different tissues.

Fig. S3 Validation of transient silencing and overexpression of tomato ERF68 in tomato H7996.

Fig. S4 Silencing of ERF68 does not affect tomato defence response to Pseudomonas syringae pv. tomato DC3000.

Fig. S5 Silencing of ERF68 in susceptible tomato L390 leads to delayed bacterial wilt caused by Ralstonia solanacearum.

Fig. S6 Validation of ERF68 binding to the promoters of the target genes by chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR) analysis.

Fig. S7 A hypothesized model for the involvement of ERF68 in incompatible tomato–Xanthomonas interactions.

Dataset S1 ChIP DNA negative peaks.

Dataset S2 ChIP DNA peaks.

Dataset S3 ChIP DNA peaks annotation.

Table S1 Summary of chromatin immunoprecipitation combined with high-throughput sequencing (ChIP-seq) assay in Nicotiana benthamiana leaves with induced transient expression of ERF68-3 × HA.

Table S2 Summary of annotated scaffolds with putative ERF68-binding region on promoters or transcriptional start sites.

Table S3 Primers and probes used in this study.